

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
25 July 2002 (25.07.2002)

PCT

(10) International Publication Number
WO 02/056879 A1

(51) International Patent Classification⁷: **A61K 31/11**,
31/405 // (A61K 31/11, 31:045)

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(21) International Application Number: **PCT/GB02/00015**

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(22) International Filing Date: 3 January 2002 (03.01.2002)

(25) Filing Language: English

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(26) Publication Language: English

(30) Priority Data:

0100059.5 3 January 2001 (03.01.2001) GB
0108246.0 2 April 2001 (02.04.2001) GB
60/326,818 4 October 2001 (04.10.2001) US

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 02/056879 A1

(54) Title: USE OF TERPENES FOR THE TREATMENT OF DIGESTIVE TRACT INFECTIONS

(57) Abstract: The prevention and treatment of digestive tract infections in humans and animals by orally administering a single terpene, a terpene mixture or a liposome-terpene (s) composition before or after the onset of a gastro-intestinal infection is described. Such infections may include traveller's diarrhea, ulcers, anthrax and other bacterial and parasitical infections.

USE OF TERPENES FOR THE TREATMENT OF DIGESTIVE TRACT INFECTIONS

1

2

3

4 The present invention relates to the treatment of
5 microbial infections, especially the prevention and
6 treatment of digestive tract infections in humans
7 and animals, by orally administering a single
8 terpene, a terpene mixture or a liposome-terpene(s)
9 composition before or after the onset of the
10 infection.

11

12 Digestive tract infections are mainly caused by
13 pathogenic and opportunistic microorganisms and
14 toxins produced by them. These illnesses are
15 present in all types of animals and humans.

16

17 Diseases caused by organisms pathogenic to humans
18 and animals are very common and encompass a range
19 from the trivial to the lethal. With the arrival
20 of the so-called 'antibiotic age' following World
21 War II, it was hoped that the scourge of infection

1 would be largely controlled on a permanent basis.
2 However, this has not proved to be the case and in
3 recent years many formerly useful prior art
4 anti-bacterials have become ineffective as
5 resistance has emerged. In the case of fungal
6 infections the armamentarium has always been
7 limited and the need remains for additional and
8 more effective treatments.

9

10 In recent years, a number of particularly difficult
11 problems have emerged and these have engaged
12 considerable public concern. For instance, the
13 rapidly rising prevalence of multiply resistant
14 *Staphylococcus aureus* (MRSA) in hospitals in
15 Western countries which has led to many deaths and,
16 to all intents and purposes, only Vancomycin now
17 stands as a fall-back treatment. Another example
18 is outbreaks of severe *E. coli* infection, such as
19 that in Scotland in the late nineteen-nineties
20 which killed over 150 people. In the case of *E.*
21 *coli*, there are particular problems in respect of
22 treatment in that, even if the organism is killed
23 quickly, the patient may die as the result of
24 endotoxins being released from the organism if it
25 is lysed as a result of anti-microbial attack.

26

27 Not all the mechanisms governing the emergence of
28 resistance to anti-bacterials are understood but
29 sufficient is known to suggest strongly that whilst
30 a fairly simple game of molecular roulette will
31 produce new anti-bacterials, any such product will

1 not remain free of resistance for long. Thus, it
2 would appear that any solution to this apparently
3 intractable problem of reduced effectiveness in
4 anti-bacterials would need to be radically
5 different to those employed in the prior art.

6

7 Recently with the scare of bio-terrorism there has
8 been an increased concern with pathogens that can
9 produce deadly outbreaks. This is the case with
10 anthrax. Anthrax is considered a potential agent
11 for use in biological warfare. Anthrax is an acute
12 infectious disease caused by the spore-forming
13 bacteria *Bacillus anthracis*. Anthrax is primarily
14 a disease of domesticated and wild animals,
15 particularly herbivorous animals. Humans become
16 infected with anthrax by handling products from
17 infected animals or by inhaling anthrax spores from
18 contaminated animal products. Anthrax can also be
19 spread by eating undercooked meat from infected
20 animals. Anthrax infection can occur in three
21 forms: cutaneous, inhalation, and gastrointestinal.
22 The most common form is the cutaneous anthrax
23 infection, which occurs when bacteria enter a cut
24 or abrasion on the skin. This infection begins as
25 a raised itchy bump that develops into a vesicle
26 and then a painless ulcer, usually 1-3 cm in
27 diameter, with a characteristic black necrotic area
28 in the center. About 20% of untreated cases of
29 cutaneous anthrax result in death. Deaths may be
30 prevented with prompt antimicrobial treatment. The
31 inhalation form has early symptom similar to a

1 common cold which progressively results in severe
2 breathing problems. This type of anthrax is
3 usually fatal. The intestinal form is
4 characterized by an acute inflammation of the
5 intestinal tract. The initial signs are nausea,
6 loss of appetite, vomiting, and fever followed by
7 abdominal pain, vomiting of blood and severe
8 diarrhea. Intestinal anthrax results in death in
9 25% to 60% of cases. Anthrax is treated with
10 antimicrobials and can be prevented with
11 vaccination. The Department of Defense in the USA
12 has a mandatory anthrax vaccination of all active
13 military personnel.

14

15 Another digestive infection in humans is
16 traveller's diarrhea, which affects over seven
17 million visitors to high-risk tropical and
18 semitropical areas every year. Others suggest that
19 the incidence of traveller's diarrhea is 15 - 56%
20 among international travelers. Approximately 1% of
21 the sufferers are hospitalized, at least 20% are
22 confined to bed for a day and nearly 40% have to
23 change plans in their travel itinerary.

24

25 Traveler's diarrhea, defined as the passage of more
26 than 3 unformed stools in a 24-hour period, is a
27 self-limiting illness lasting 3 - 5 days. The
28 illness may be presented either as (1) acute watery
29 diarrhea (2) diarrhea with blood (dysentery) or (3)
30 chronic diarrhea, often with clinical nutrient
31 malabsorption.

1 Several factors contribute to the development of
2 diarrhea in travelers, including personal (age,
3 socioeconomic status, body weight, preexisting
4 gastrointestinal illnesses), behavioral (mode of
5 travel, standard of accommodation, eating in public
6 places, dietary errors) and travel related
7 (destination, duration of stay, country of origin,
8 season). Approximately 85% of the diarrheas
9 among international travelers are produced by
10 bacterial enteropathogens. These pathogens are
11 usually acquired through ingestion of fecally
12 contaminated food or water. Sometimes dirty hands
13 or insects are the vectors of fecal contamination.
14 Cooked food is safe to consume as long as the
15 temperature at the interior of the food reaches
16 160°F or more. An undercooked hamburger is risky
17 food, because ground meat can become contaminated
18 at the processing plant and during preparation.
19
20 The common pathogens that produce traveler's
21 diarrhea include *Clostridium difficile*, *Yersenia*
22 *enterolitica*, *Shigella* sp., *Campylobacter* sp.,
23 *Salmonella* sp., ETEC (enterotoxigenic) and EAEC
24 (enteroaggregative) *Escherichia coli*. Traveler's
25 diarrhea produced by *Shigella* sp. or *Salmonella* sp.
26 tend to cause a more severe and longer lasting
27 disease than that caused by the most common cause,
28 enterotoxigenic *E. coli* (ETEC). *Campylobacter*
29 *jejuni* is a relatively common cause of traveler's
30 diarrhea especially in the winter. Viruses such as

1 rotavirus, cytomegalovirus and Norwalk agent are
2 less common causes.

3

4 There are several groups of pathogenic *E. coli*.
5 They include Enterotoxigenic (ETEC), which produce
6 a range of toxins, heat-stable or heat-labile in
7 nature. ETEC is the most common cause of diarrhoea
8 disease in children in the developing world; it
9 also causes many travelers' diarrhoea cases.

10

11 Verocytotoxic *E. coli* (VTEC) strains produce toxins
12 that destroy the gut mucosa and can cause kidney
13 damage; *E. coli* 0157 H:7 is the most publicised
14 example of this type.

15

16 Enteropathogenic *E. coli* (EPEC) do not appear to
17 produce toxins but may attach the microvilli, this
18 group often causes infection in babies and young
19 children.

20

21 Enteroinvasive *E. coli* (EIEC) attaches to the
22 mucosal lining of the large intestine and invade
23 the cells, causing tissue destruction and
24 inflammation. EIEC are usually food borne
25 pathogens and are an important cause of disease in
26 areas of poor hygiene.

27

28 The severity of the disease symptoms are dependent
29 on the strain encountered and the underlying health
30 of the individual. EIEC and VTEC strains can cause
31 very serious disease (haemorrhagic colitis and

1 renal failure) and require hospitalisation. Milder
2 cases are usually treated by fluid and electrolyte
3 replacement and rest.

4

5 The use of antibiotics limits the course of
6 diarrhea to a little over a day compared with an
7 average of over 3 - 5 days when diarrhea remains
8 untreated. The widespread resistance of the
9 traditional antimicrobial agent, Trimethoprim plus
10 sulfamethoxazole (TMP/SMX), and fluoroquinolones
11 are the main reasons of concern about the
12 continuous use of antimicrobials for the treatment
13 of traveler's diarrhea (Dupont et al, 1998). The
14 extensive use of antibiotics can also lead to
15 overgrowth syndromes, *Candida vaginitis* can occur,
16 the overgrowth of *Clostridium difficile* due to less
17 competitive environment in the gastrointestinal
18 tract can also result in diarrhea.

19

20 Short-term travelers that have experience diarrhea
21 do not develop protection, since it requires
22 continued exposure to enteropathogens to develop
23 immunological protection against traveler's
24 diarrhea. Vaccination is a promising option, but
25 vaccines against all enteropathogens that cause
26 traveler's diarrhea have not been developed. Other
27 protection methods to treat traveler's diarrhea
28 are: the use of nonabsorbed antimicrobials, which
29 have fewer side effects and should be safer to use
30 in children and pregnant women in whom quinolones
31 are contraindicated; antisecretory and antimotility

1 agent (loperamide); the use of attapulgite, a
 2 hydrated aluminum silicate clay preparation; and
 3 probiotics i.e. lactobacillus, which appear to be
 4 useful in the prevention or treatment of travelers
 5 diarrhea. In all cases the restoration of water
 6 and electrolyte balance is necessary. The following
 7 table shows the current treatments for Traveler's
 8 Diarrhea:

9

Agent	Efficacy	Comments
Activated charcoal	Not efficacious	May absorb important medications
Lactobacillus	Not proven	Safe
Bismuth subsalicylate preparations	65% protective	Rinse mouth to avoid black tongue
Trimethoprim-Sulfamethoxazole	70-80% protective	Resistance rising worldwide
Fluoroquinolones (norfloxacin, ciprofloxacin, Ofloxacin)	90% protective or better	Currently most effective antimicrobial but resistance rising worldwide.

10 Ericsson, Charles (1998)

11

12 In humans and animals, peptic ulcers are open sores
 13 produced by a bacteria. These open sores can be
 14 present on the entire gastro-intestinal tract,
 15 mainly esophagus, stomach and proximal part of the
 16 small intestine. There is evidence that support
 17 the role of *H. pylori* as the etiologic agent of
 18 chronic gastritis and peptic ulcer. *H. pylori*, a

1 gram-negative, microaerophilic spiral bacteria is
2 the major cause of gastro-duodenal disease,
3 including chronic gastritis, gastric and duodenal
4 ulcers and gastric neoplasia. Greater than 50% of
5 North American adults over 50 years of age are
6 infected with *H. pylori*. In contrast, in some
7 developing and newly industrialized countries
8 virtually all adults are infected. In developing
9 countries almost all children are infected by age
10 10, whereas in developed countries only the
11 children of lower socioeconomic levels are
12 infected. *H. pylori* is characterized by very high
13 urease activity that may be associated with
14 virulence, in the absence of urea *H. pylori* is
15 sensitive to acidic pH. Urease activity may be an
16 important colonization and survival factor by
17 generating ammonia in the immediate bacterial
18 microenvironment. *H. pylori* has been classified as
19 a type 1 carcinogen by the World Health
20 Organization because of the danger of persistent
21 infection with the bacterium causing gastric
22 cancer. *H. pylori* infection is of extreme
23 importance in the causation of peptic ulcer
24 disease. By initiating a gastritis or dyspeptic
25 symptoms, it can predispose to subsequent episode
26 of either gastric lymphoma or stomach cancer.
27
28 The eradication of *H. pylori* has been obtained with
29 combination therapy, triple therapy using bismuth
30 plus two antibiotics (metronidazole and either
31 amoxicillin or tetracycline has been effective).

1 Problems due to development of antimicrobial
2 resistant and side effects (diarrhea, nausea,
3 abdominal pain and others) may explain why the use
4 of antibiotics has not become a preferred treatment
5 for gastritis and peptic ulcers due to *H. pylori*.

6

7 Antibacterial treatment of *H. pylori* is difficult
8 because of the habitat occupied by the organism
9 below the layer of the mucus adherent to the
10 gastric mucosa. Access of antibacterial agents to
11 this site is limited from the lumen of the stomach
12 and also from the gastric blood supply.

13

14 The use of medium chain fatty acids and medium
15 chain triglycerides has been shown to inhibit the
16 growth of *H. pylori* in vitro. The mechanism by
17 which they exert antibacterial effect is thought to
18 involve: 1) damage to the bacterial outer membrane
19 leading the increase membrane fluidity and
20 permeability, 2) Incorporation of these fatty
21 acids, making the bacterial membrane unstable, 3)
22 Production of peroxides due to oxidation of fatty
23 acids.

24

25 The mode of transmission of *H. pylori* in humans is
26 still poorly understood. There are reports of
27 detection of this microorganism in the oral cavity
28 and in the feces. If *H. pylori* is harvested in the
29 oral cavity or bowel, these might represent
30 important reservoir for the reinfection and
31 transmission with consequences from treatment. One

1 vector for the transmission of *H. pylori* are flies,
2 they can carry viable *H. pylori* in their external
3 surfaces and alimentary tracts.

4

5 In animals, the presence of scours in calves is of
6 economic importance. It is estimated that the
7 death lost of calves less than 6 months of age is
8 approximately 2.5% or over 100,000 a year. Most of
9 the mortality and morbidity of the calves are due
10 to infectious diseases, mainly scours. More than
11 90% of scours in calves is produced by *E. coli* and
12 *Salmonella*. *Clostridia* has proved to be fatal in
13 the majority of cases. There are preventive
14 methods like (1) vaccination of the mothers in
15 order to passively transfer antibodies in
16 colostrum; (2) the use of immunological supplements
17 for milk replacers; (3) the use of probiotics to
18 create a gastro-intestinal healthy environment (4)
19 changes in calf management. None of these
20 protective measures are 100% effective.

21

22 Another animal of economic importance is swine.
23 The incidence of diarrhea in neonates and weaned
24 piglets is very high. Again, *E. coli* and
25 *Salmonella* are the main microorganisms involved in
26 diarrhea in swine. There are losses in the nursery
27 while piglets are still lactating and after
28 weaning. There are similar preventive methods as
29 in calves. One of the preferred methods is
30 segregated early weaning (SEW). The basis of early
31 weaning is that the earlier piglets are weaned from

1 the sow the less are the chances of crossover
2 diseases between sow and piglets. This method
3 requires the use of antibiotics.

4

5 In both cases, calf and piglet scours, the
6 preferred method of treatment is antibiotics. The
7 European Community has banned the use of 5
8 antibiotics and in the United States the FDA is
9 banning the use of fluoroquinolone in animals due
10 to the development of *Campylobacter* resistant to
11 this antibiotic. Bacteria resistance has
12 encouraged the development of antibiotic-
13 alternative products.

14

15 Terpenes are widespread in nature, mainly in plants
16 as constituents of essential oils. Their building
17 block is the hydrocarbon isoprene (C_5H_8)_n. Terpenes
18 have been found to be effective and nontoxic
19 dietary antitumor agents which act through a
20 variety of mechanisms of action (Crowell and Gould,
21 1994 and Crowell et al, 1996). Terpenes, i.e.
22 geraniol, tocotrienol, perillyl alcohol, b-ionone
23 and d-limonene, suppress hepatic HMG-COA reductase
24 activity, a rate limiting step in cholesterol
25 synthesis, and modestly lower cholesterol levels in
26 animals (Elson and Yu, 1994). D-limonene and
27 geraniol reduced mammary tumors (Elegbede et al,
28 1984 and 1986 and Karlson et al, 1996) and
29 suppressed the growth of transplanted tumors (Yu et
30 al, 1995).

31

1 Terpenes have also been found to inhibit the
2 in-vitro growth of bacteria and fungi (Chaumont and
3 Leger, 1992, Moleyar and Narasimham, 1992 and
4 Pattnaik, et al, 1997) and some internal and
5 external parasites (Hooser, et al, 1986). Geraniol
6 was found to inhibit growth of *Candida albicans* and
7 *Saccharomyces cerevisiae* strains by enhancing the
8 rate of potassium leakage and disrupting membrane
9 fluidity (Bard, et al, 1988). B-ionone has
10 antifungal activity which was determined by
11 inhibition of spore germination, and growth
12 inhibition in agar (Mikhlin et al, 1983 and Salt et
13 al, 1986). Teprenone (geranylgeranylacetone) has
14 an antibacterial effect on *H. pylori* (Ishii, 1993).
15 Solutions of 11 different terpenes were effective
16 in inhibiting the growth of pathogenic bacteria in
17 in-vitro tests; levels ranging between 100 ppm and
18 1000 ppm were effective. The terpenes were diluted
19 in water with 1% polysorbate 20 (Kim et al, 1995).
20 Diterpenes, i.e. trichorabdal A (from R.
21 Trichocarpa) has shown a very strong antibacterial
22 effect against *H. pylori* (Kadota, et al, 1997).
23
24 Rosanol a commercial product with 1% rose oil has
25 been shown to inhibit the growth of several
26 bacteria (*Pseudomonas*, *Staphylococcus*, *E. coli* and
27 *H pylori*). Geraniol is the active component (75%)
28 of rose oil. Rose oil and geraniol at a
29 concentration of 2 mg/litre inhibited the growth of
30 *H pylori* in vitro. Some extracts from herbal
31 medicines have been shown to have an inhibitory

1 effect on *H. pylori*, the most effective being
2 decursinol angelate, decursin, magnolol, berberine,
3 cinnamic acid, decursinol and gallic acid (Bae, et
4 al 1998). Extracts from cashew apple, anacardic
5 acid and (E)-2-hexenal, have shown bactericidal
6 effect against *H. pylori*.

7 There may be different modes of action of terpenes
8 against *H. pylori*. They could (1) interfere with
9 the phospholipid bilayer of the cell membrane (2)
10 impair a variety of enzyme systems (HMG-reductase)
11 and (3) destroy or inactivate genetic material.

12

13 SUMMARY OF THE INVENTION

14

15 Prevention and treatment of digestive tract
16 infections by orally administering a biocidal
17 terpene, a biocidal terpene mixture or a
18 liposome-terpene(s) composition before or after the
19 onset of the infection.

20

21 DESCRIPTION OF THE PREFERRED EMBODIMENTS

22

23 Digestive tract infections not only are an
24 uncomfortable illness for humans but also are of
25 economic importance for the animal industry. In
26 some cases the illness can cause death in children,
27 elderly and immune-compromised people. The
28 preferred treatment of the disease is antibiotics.
29 The extensive use of antibiotics in humans and the
30 animal industry has created the development of
31 antibiotic-resistant bacteria. The increased

1 antibiotic resistance has been the main reason to
2 seek new antimicrobial alternatives. The European
3 Community has banned the use of 5 antibiotics in
4 animals and in the United States the FDA is banning
5 the use of fluoroquinolone in animals due to the
6 development of *Campylobacter* resistant to this
7 antibiotic.

8 Terpenes, which are GRAS (Generally Recognized As
9 Safe) have been found to inhibit the growth of
10 cancerous cells, decrease tumor size, decrease
11 cholesterol levels and have a biocidal effect on
12 microorganisms in vitro. Onawunmi (1989) showed
13 that growth media with more than 0.01 % citral
14 reduced the concentration of *E. coli* and at 0.08%
15 there was a bactericidal effect. Barranx, et al
16 (1998) teach us a terpene formulation, based on
17 pine oil, used as a disinfectant or antiseptic
18 cleaner. Koga, et al (1998) teach that a terpene
19 found in rice has antifungal activity. Iyer, et al
20 (1999) teach us an oral hygiene antimicrobial
21 product with a combination of 2 or 3 terpenes that
22 showed a synergistic effect. Neither of them
23 suggested the use of a terpene, terpene mixture or
24 liposome-terpene(s) combination for the prevention
25 or treatment of gastro-intestinal infections i.e.
26 traveler's diarrhea.

27

28 Several US Patents (US#5,547,677, US#5,549,901,
29 US#5,618,840, US#5,629,021, US#5,662,957,
30 US#5,700,679, US#5,730,989) teach us that certain

1 types of oil-in-water emulsions have antimicrobial,
2 adjuvant and delivery properties.

3

4 Thus, the present invention provides a composition
5 for preventing or treating gastro-intestinal
6 infections, wherein said composition comprises a
7 terpene or a mixture of terpenes. We have found
8 that certain mixture of terpenes are
9 synergistically effective, relative to the effects
10 of the component terpenes administered separately.
11 Thus terpenes having biocidal activity which in
12 combination with two or more other terpenes
13 synergistically increase the biocidal effectiveness
14 are of especial interest.

15

16 One composition of interest comprises a mixture of
17 carvone and geraniol, optionally together with
18 other terpenes. The content of carvone and
19 geraniol may each be from 10 to 90% (by weight),
20 but is preferably 10 to 60% by weight. Other
21 terpenes which may be present include citral, b-
22 ionone, eugenol, terpeniol, carvacrol, anethole or
23 the like. These optional additional terpenes may
24 be present at 5 to 50% by weight, for example 10 to
25 40% by weight.

26

27 Optionally, the terpenes may be presented in the
28 form of liposomes.

29

30 Liposomes are microscopic structures consisting of
31 concentric lipid bilayers enclosing an aqueous

1 space. Liposomes are classically prepared from
2 phospholipids which occur naturally in animal cell
3 membranes, but several synthetic formulations are
4 now commonly used. The lipid composition of the
5 liposome can be varied to give liposomes different
6 physical characteristics i.e. size and stability.
7 Liposomes can be prepared by the reverse-phase
8 evaporation or dehydration-rehydration vesicle
9 methods using a mixture of dipalmitoyl phosphatidyl
10 choline, cholesterol, dipalmitoyl phosphatidyl
11 glycerol, dipalmitoyl phosphatidyl ethanolamine and
12 other synthetic fatty acids and emulsifiers. When
13 making liposomes first multilamellar vesicles are
14 formed spontaneously when amphipathic lipids are
15 hydrated in an aqueous medium. Unilamellar vesicles
16 are often produced from multilamellar vesicles by
17 the application of ultrasonic waves.

18

19 Multilamellar vesicles can be prepared by the
20 procedure known as dehydration-rehydration.
21 Briefly, egg phosphatidylcholine and cholesterol
22 are mixed in chloroform, dried in a rotary
23 evaporator, dilute with water and sonificated to
24 form unilamellar vesicles. The solution is freeze
25 dried and rehydrated with the terpene solution in
26 order to embed the terpene inside the liposome.
27 Another method to produce liposomes is by mixing
28 together lipids, an emulsifier and the terpenes.
29 The emulsion is obtained by using a Polytron
30 homogenizer with special flat rotor that creates an
31 emulsion. The lipids could consist of soybean oil,

1 any commercial or pharmaceutical oil; the
2 emulsifier consist of egg yolk lecithin, plant
3 sterols or synthetic including polysorbate-80,
4 polysorbate-20, polysorbate-40, polysorbate-60,
5 polyglyceryl esters, polyglyceryl monooleate,
6 decaglyceryl monocaprylate, propylene glycol
7 dicaprilate and triglycerol monostearate. The
8 lipid concentration in the oil phase is 75-95% and
9 the emulsifier concentration from 5-25%. When
10 preparing the emulsion a ratio oil to water could
11 vary from 10-15 parts lipid to 35-40 parts terpenes
12 diluted in water at a concentration of 0.5% to 50%.
13 Once the emulsion is formed this is combined with a
14 carrier in order to be use as a humectant, cream or
15 other suitable carrier for topical applications.
16 The emulsion concentration use for topical
17 application varies from 0.0055 through 1.0% of the
18 final product. Several modifications to the
19 emulsion can be achieved by simply varying the
20 concentration and type of terpenes used. This
21 modification can give us different products with
22 different antimicrobial specificity.
23
24 By encapsulating terpenes within these emulsions
25 the antimicrobial effect will be increased: (1) the
26 liposome will disrupt the bacterial membrane and
27 (2) the terpenes will be more effective in
28 disrupting cytoplasmatic enzymes.
29
30 It will be apparent for those skilled in the art
31 that the aforementioned objects and other

1 advantages may be further achieved by the practice
2 of the present invention.

3

4 EXAMPLE 1: Preparation of the terpene mixture

5

6 The terpene, terpene mixture or liposome-terpene(s)
7 combination consists of a blend of generally
8 recognized as safe (GRAS) terpenes with a GRAS
9 surfactant. The ratio of terpenes is from 1-99%
10 and the surfactant ratio from 1-99% of the mixture.
11 The terpenes, comprised of natural or synthetic
12 terpenes, are citral, b-ionone, geraniol, eugenol,
13 carvone, terpeniol, carvacrol, anethole or other
14 terpenes with similar properties. The surfactant is
15 preferably polysorbate-80 or other suitable GRAS
16 surfactants.

17

18 EXAMPLE 2: Preparation of liposomes containing
19 terpenes

20

21 Any standard method for the preparation of
22 liposomes can be followed with the knowledge that
23 the lipids used are all food-grade or
24 pharmaceutical-grade. A set amount of lipids, an
25 emulsifier and the terpenes was used to prepare an
26 emulsion. The emulsion was obtained by using a
27 Polytron homogenizer with special flat rotor that
28 created an emulsion. The lipids consisted of
29 soybean oil, any commercial or pharmaceutical oil;
30 the emulsifier consist of egg yolk lecithin, plant
31 sterols or synthetic emulsifiers including

1 polysorbate-80, polysorbate-20, polysorbate-40,
2 polysorbate-60, polyglyceryl esters, polyglyceryl
3 monooleate, decaglyceryl monocaprylate, propylene
4 glycol dicaprilate and triglycerol monostearate. A
5 solution containing 75-95% lipids (oil) and 5-25%
6 emulsifier consisted of the oil phase. The aqueous
7 phase consisted of the terpene diluted in water at
8 a rate of 0.5% to 50%. To form the emulsion a
9 ratio of oil to water varying from 10-15 parts
10 lipid (oil phase) to 35-40 parts terpenes (aqueous
11 phase) was mixed. Any standard method for the
12 preparation of liposomes can be followed with the
13 knowledge that the lipids used are all food-grade
14 or pharmaceutical-grade. The suspension containing
15 a lipid, an emulsifier and the terpenes is
16 emulsified with a Posytron homogenizer until a
17 complete milky solution is obtained.

18

19 EXAMPLE 3: Preparation of liposomes

20

21 This step consists of the preparation of the
22 terpene(s)-liposome combination by mixing 99% of
23 liposome and 1% of terpene mixture. Several
24 combinations of this formulation can be obtained by
25 varying the amount of terpene and liposome from 1%
26 to 99%. The liposomes are prepared as in Example 2
27 without the addition of terpenes in the
28 formulation.

29

30 EXAMPLE 4: In-vitro effectiveness of terpenes
31 against E. coli

1 This example demonstrates the effect of terpenes on
2 the cell membrane fragility of *E. coli*, which is
3 considered indicative of other pathogenic bacteria
4 such as *Salmonella* and *Listeria*. Lysis of the cell
5 membrane was monitored by the determination of
6 galactosidase activity. β -galactosidase is a
7 well-characterized cytosolic enzyme in bacteria.
8 This enzyme is inducible in the presence of
9 isopropyl-1-thiogalactosidase (IPTG) and assayed
10 colorimetrically with substrate
11 o-nitro-phenyl- β -D-galactoside (ONPG). ONPG is
12 cleaved to release o-nitrophenol with peak
13 absorbance at 420 nm. Since intact *E. coli* is
14 impermeable to both ONPG and the enzyme, the cells
15 have to be lysed prior to enzymatic assay.
16 Therefore the ability of terpenes to lyse *E. coli*
17 can be measured with this enzymatic assay and
18 compared to known lysing agents.

19

20 The procedure used was as follows: *E. coli* strains
21 AW574 or AW405 were cultured overnight in 10 ml
22 tryptone broth with 1 nM IPTG at 35°C. Cells were
23 allowed to grow until an absorbance equal to 0.9
24 was reached. Cells were harvested, washed with
25 phosphate buffer and resuspended to an absorbance
26 equal to 0.5. 0.1 ml of the bacteria culture was
27 added to 0.9 ml of buffer, warmed to 30°C and then
28 80 μ l of terpenes (85% terpenes and 15%
29 polysorbate-80), 80 μ l water (background) or 40 μ l
30 chloroform plus 40 μ l 1% SDS in water (positive

1 control) were added. After the addition of the
2 lysing agents the tubes were mixed for 10 seconds
3 and 0.2 ml of ONPG (4 mg/ml water) was added, then
4 incubated for 5 minutes. The enzyme activity was
5 stopped with 0.5 ml of 1 M sodium carbonate. After
6 being centrifuged for 3 minutes at 1,500 x g,
7 supernatant was transferred to cuvettes and read at
8 420 nm. The relative degree of lysis caused by
9 terpenes was calculated as follows:

10
11 100 x (OD terpenes-OD water) / (OD chloroform-OD
12 water)

13
14 This shows that dosages can be manipulated to
15 either lyse the cell outright, or in the case of
16 lower dosages, stop bacterial growth without lysis
17 of the cell membrane. The advantage of this
18 controllable result is the ability to prevent lysis
19 and the resultant release of endotoxins where
20 contraindicated.

21

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1 Table 1: Lysis of *E. coli* by Terpenes

Terpenes (μM)		Relative lysis %
Carvone	404,000	NM*
	40,400	54
	4,040	22
	404	3.2
Geraniol	363,000	NM
	36,300	96
	3,630	98
	363	34
	36.3	4
	3.63	2.4
β -Ionone	308,000	NM
	30,800	NM
	3,080	NM
	308	52
	30.8	44
	3.08	23
	0.308	4.78
	0.0308	1.3
80 μl Polysorbate-80		3.2
80 μl Polysorbate-80 + SDS + Chloroform		100
SDS + Chloroform		100*

2

3 *Lysis due to chloroform and SDS combination was
 4 considered to be 100%.

5 *NM, not measurable due to formation of turbid
 6 colloidal solution.

1 EXAMPLE 5: In in-vitro effectiveness of terpenes
2 against several microorganisms

3

4 This example demonstrate the effectiveness of
5 terpenes against *Escherichia coli*,
6 *Salmonella typhimurium*, *Pasteurella mirabilis*,
7 *Staphylococcus aureus*, *Candida albicans* and
8 *Aspergillus fumigates*. Each organism, except *A.*
9 *fumigatus*, was grown overnight at 35-37°C in
10 tryptose broth. *A. fumigatus* was grown for 48
11 hours. Each organism was adjusted to approximately
12 10^5 organisms/ml with sterile saline. For the
13 broth dilution test, terpenes were diluted in
14 sterile tryptose broth to give the following
15 dilutions: 1:500, 1:1000, 1:2000, 1:4000, 1:8000,
16 1:16,000, 1:32,000, 1:64,000 and 1:128,000.

17

18 Each dilution was added to sterile tubes in 5 ml
19 amounts. Three replicates of each series of
20 dilutions were used for each test organism. 0.5 ml
21 of the test organism was added to each series and
22 incubated at 35-37°C for 18-24 hours. After
23 incubation the tubes were observed for growth and
24 plated onto blood agar. The tubes were incubated
25 an additional 24 hours and observed again. The *A.*
26 *fumigates* test series was incubated for 72 hours.
27 The minimum inhibitory concentration for each test
28 organism was determined as the highest dilution
29 that completely inhibits the organism.

30

1 Table 2: Results of the inhibitory activity of
 2 different dilutions

Organism	Visual assessment of growth *			Growth after subculture to agar plates *			Mean inhibitory dilution
	1	2	3	1	2	3	
<i>S. typhimurium</i>	500	500	500	500	500	500	500
<i>E. coli</i>	1000	1000	1000	1000	1000	1000	1000
<i>P. mirabilis</i>	1000	1000	1000	1000	1000	1000	1000
<i>S. aureus</i>	1000	1000	1000	1000	1000	1000	1000
<i>C. albicans</i>	1000	1000	1000	1000	1000	1000	1000
<i>A. fumigatus</i>	8000	16000	16000	8000	16000	16000	13300

* The results of the triplicate tests with each organism as the reciprocal of the dilution that showed inhibition/killing

** NI = not inhibited

3

4

5 EXAMPLE 6: In in-vitro effectiveness of terpenes
 6 against Escherichia coli over time.

7

8 This example demonstrates the effectiveness of
 9 terpenes at several concentrations against
 10 *Escherichia coli* and cultured over time. Terpene
 11 dilutions (1:500, 1:1000, 1:2000, 1:4000, 1:8000,
 12 and 1:16,000) were prepared in BHI broth and in
 13 saline. These were prepared in 25 ml amounts. *E.*
 14 *coli* was grown overnight in BHI broth and diluted
 15 to a MacFarland 0.5 concentration in saline. This
 16 solution was diluted 1:100 to be used to inoculate

1 (0.5 ml) each terpene dilution tube. The series
 2 that contained the terpene dilution in BHI was
 3 tested at 30 min, 90 min, 150 min and 450 min.
 4 Each tube was mixed and serially diluted in saline.
 5 0.5 ml of each dilution was spread plated onto
 6 MacConkey (MAC) agar plates. Also, 3 drops of the
 7 undiluted and the 1:100 dilution was added into
 8 respective tubes of BHI broth. The tubes and
 9 plates were incubated overnight at 35°C. The
 10 series that contained the terpene's dilution in
 11 saline were tested at 60 min, 120 min, 180 min and
 12 480 min. Each tube was mixed and serially diluted
 13 in saline. 0.5 ml of each dilution was spread
 14 plated onto MacConkey (MAC) agar plates. Also, 3
 15 drops of the undiluted and the 1:100 dilution were
 16 added into respective tubes of BHI broth. The
 17 tubes and plates were incubated overnight at 35°C.
 18

19 Table 3: Subculture from the tubes containing
 20 various dilutions of terpenes in broth

Time	Dilution	1:500	1:1000	1:2000	1:4000	1:8000	1:16,000
30 min	Undiluted	NG	+	+	+	+	+
	1:100	NG	+	+	+	+	+
90 min	Undiluted	NG	NG	+	+	+	+
	1:100	NG	NG	NG	+	+	+
150 min	Undiluted	NG	NG	+	+	+	+
	1:100	NG	NG	NG	+	+	+
450 min	Undiluted	NG	NG	+	+	+	+
	1:100	NG	NG	+	+	+	+

21 NG: no growth, +: growth

1 Table 4: Subculture from the tubes containing
 2 various dilutions of terpenes in saline
 3

Time	Dilution	1:500	1:1000	1:2000	1:4000	1:8000	Control
60 min	Undiluted	NG	+	+	+	+	+
	1:100	NG	NG	NG	+	+	+
120 min	Undiluted	NG	NG	NG	+	+	+
	1:100	NG	NG	NG	NG	+	+
180 min	Undiluted	NG	NG	NG	+	+	+
	1:100	NG	NG	NG	NG	+	+
480 min	Undiluted	NG	NG	NG	NG	+	+
	1:100	NG	NG	NG	NG	NG	+

4 NG: no growth, +: growth

5

6 Table 5: The quantitative results of the activity
 7 of various terpene dilutions against *E.coli*
 8 (cfu)

Media	Time	1:500	1:1000	1:2000	1:4000	1:8000	Control
Broth	30 min	0	0	660	3600	3600	4600
	90 min	0	0	12	4600	5400	7600
	150 min	0	0	10	8000	12,000	14,000
	450 min	0	0	15,000	28×10^3	23×10^7	16×10^8
Saline	60 min	0	4	140	4000	2000	1300
	120 min	0	0	0	90	3800	2600
	180 min	0	0	0	2	2000	5000
	480 min	0	0	0	0	104	8000

9 NG: no growth, +: growth

10

11

12

1 EXAMPLE 7: In vitro effectiveness of selected
2 terpenes on *Helicobacter pylori*.

3

4 This example shows the bactericidal effect of
5 selected terpenes on the viability of *H. pylori*.
6 Five terpenes (anethole, carvone, citral, geraniol
7 and b-ionone) were used for this study. Terpenes
8 were mixed to a ratio of 90% terpene plus 10%
9 polysorbate-80. The *H. pylori*, used was strain
10 #26695 of porcine origin, this bacteria is a
11 motile, cag A, vac A cytotoxin-positive gram
12 negative bacteria which colonizes gnotobiotic
13 piglets and indefinitely persists within the
14 gastric microenvironment as a superficial infection
15 of the gastric mucosa and mucus layer.

16

17 The study was as follows:

18

19 1) Stock solutions of each terpene with
20 polysorbate-80 were prepared (1.8 ml terpene plus
21 0.2 ml polysorbate-80).

22

23 2) Stock solutions were diluted in Brucella broth
24 10% (v/v) fetal calf serum to a final concentration
25 of stock at 1:10, 1:50, 1:100, 1:500, 1:1000,
26 1:5000 and 1:10000. Controls consisted of 10%
27 (v/v) polysorbate-80 in Brucella broth, Brucella
28 broth alone and bacteria in Brucella broth.

29

30 3) A total of 1.0×10^6 bacteria (30 μ l) was added
31 to 970 μ l terpene dilutions (final volume of 1.0

1 ml) in loosely capped tubes and incubated for 24
2 hours at 37 °C with continuous mixing.

3

4 4) Duplicate samples (0.1 ml) from each test
5 dilution was titrated onto blood agar plates and
6 incubated for 48 hours at 37°C on 10% CO₂
7 environment. Bacterial colony forming units (cfu)
8 were determined by visual (counting) inspection.
9 Recovered bacteria were confirmed to be *H. pylori*
10 by catalase and urease enzyme activities.

11

12 The results are summarized in the following table:

13

14 Table 6: Effect of different terpenes on *H. pylori*
15 growth

	Final dilution tested for antimicrobial effects against 10 ⁶ cfu							
Terpene	1:10	1:50	1:100	1:500	1:1000	1:5000	1:10000	1:50000
Polysorbate -80	NG*	NG	NG	10 ³	10 ⁴	TNTC**	TNTC	TNTC
Anethole	NG	NG	NG	NG	10 ³	10 ³	TNTC	TNTC
Carvone	NG	NG	NG	NG	NG	10 ⁴	TNTC	TNTC
Geraniol	NG	NG	NG	NG	NG	NG	10 ²	TNTC
Citral	NG	NG	NG	NG	NG	NG	NG	TNTC
b-ionone	NG	NG	NG	NG	NG	NG	NG	TNTC

16 * NG = no growth ** TNTC = Too Numerous To Count

17

18 EXAMPLE 8: In vitro effectiveness of single or
19 combination of terpenes against *E. coli*.

20

21 The objective of this example was to determine an
22 optimum terpene mixture which could have a greater

1 biocidal effect. *E. coli* strain AW574 was grown in
2 tryptone broth to an exponential growth phase (O.D.
3 between 0.4 and 1.0 at 590 nm). One tenth of this
4 growth was inoculated to 10 ml of tryptone broth
5 followed by the addition of individual terpenes as
6 indicated in Example 5; then incubated for 24 hours
7 at 35-37°C and the O.D. determined in each tube.
8 The concentration of terpenes was 1 or 2 µMol.
9 Each treatment was repeated in triplicate. The
10 results are expressed as percentage bacterial
11 growth as compared to the control treatment. It is
12 observed that the combination of terpenes give
13 better biocidal effect than single terpenes, with
14 geraniol and carvone better than β -ionone.

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1 Table 7: Effect of single terpene or their
 2 combination against on *E. coli* growth

3

$\mu\text{Mol terpenes}$			% Growth
B-ionone	Carvone	Geraniol	
0	0	0	100.00
2	0	0	84.00
0	2	0	63.00
0	0	2	54.00
1	1	1	41.00
1	2	1	31.10
1	1	2	14.80
1	2	2	15.90
2	1	1	48.60
2	2	1	44.30
2	1	2	30.20
2	2	2	1.50

4

5 EXAMPLE 9: In vitro effectiveness of a combination
 6 of terpenes against different *E. Coli* strains

7

8 Both well-test and broth test methods were used to
 9 assess the effect of terpene formulations against a
 10 variety of strains of *E. coli*. The broth test
 11 method was judged to be a more applicable
 12 simulation of gastrointestinal tract conditions
 13 than the well plate (zone of inhibition) method. A
 14 series of broth tests was conducted on a selected
 15 test formulation to determine its activity in an
 16 aqueous environment.

17

1 **Test micro-organisms**

2

3 Bacteria were sub-cultured from original American
4 Type Culture Collection (ATCC) freeze-dried
5 material. They included *E. coli* strains 8739,
6 25922 and 700728 (Serotype group O: 157 H:7), which
7 are BioSafety class 1 organisms and *E. coli* 12795
8 (Serotype group O: 26) which is a BioSafety class 2
9 organism. All the bacteria were cultured on
10 Tryptone Soya Agar (TSA), supplied by Oxoid Ltd,
11 Hampshire and Mueller Hinton Agar (MHA), supplied
12 by Merck Ltd. The incubation temperature was
13 35 °C.

14

15 **Broth Test Procedure**

16

17 *E. coli* cultures were prepared in nutrient broth
18 and allowed to grow until exponential growth phase
19 was achieved (16 hours at 35 °C). 1 ml of this
20 culture was transferred to each of a series of
21 pre-sterilised Duran bottles containing 100 ml
22 nutrient broth, 0.5 % w/v Polysorbate 80 and this
23 gave an initial inoculum of approximately 10^8
24 microbial cells per ml of broth.

25

26 The Duran bottles were agitated on a vortex shaker
27 to produce good mixing and the Optical Density (OD)
28 at 590 nm read on a calibrated Unicam UV 300
29 spectrophotometer controlled by Vision 32 software.
30 The OD of a sample of a placebo broth was also
31 recorded.

1 The bottles were then placed in an incubator at
2 35°C. The bottles were removed at 30 minute
3 intervals and placed on a vortex shaker at level
4 three for 30 seconds. The bottles were then
5 returned to the incubator. The OD was recorded at
6 hourly intervals, for up to 24 hours.

7

8 After completion of the tests, the broths were
9 autoclaved on programme 4 of an AVX240 autoclave
10 (132 °C for 30 minutes) to sterilise them.

11

12 The terpenoids tested in this series of exemplary
13 experiments included I-carvone, citral and geraniol
14 in varying proportions. One exemplary formulation,
15 constituting the test formulation, is given in
16 Table 8, below

17

Table 8	% w/w
I-Carvone	40
Citral	40
Geraniol	15
Polysorbate 80	5
Total	100

18

19 This exemplary test formulation was highly active
20 and clear inhibition of *E. coli* growth was observed
21 in broth tests conducted at 50 µl and 100 µl doses
22 in 100 ml broth.

23

24

25

1 Table 9: *E. coli* 8739 Broth Test of Formulation
2 (Optical Density at 590 nm)

3

Time Post Inoculation	Volume of Test Formulation (μ l)					
	500	100	50	10	5	Control
0	0.958	0.063	0.019	0.007	0.007	0.003
1	0.708	0.025	0.028	0.024	0.021	0.022
2	0.762	0.023	0.053	0.081	0.087	0.102
3	0.547	0	0.094	0.158	0.179	0.191
4	0.486	0	0.140	0.270	0.286	0.323
5	0.594	0	0.181	0.316	0.311	0.345
6	0.522	0.011	0.238	0.361	0.367	0.401
6.5	0.579	0.014	0.262	0.376	0.372	0.411
23	0.617	0.031	0.285	0.619	0.747	0.654
24	0.553	0.058	0.286	0.606	0.740	0.683

4

5 The 16 hour old *E. coli* culture used as the
6 inoculum had an OD at 590 nm of 0.697 units.

7

8 Further broth tests were conducted against two
9 pathogenic strains of *E. coli* (700728, 12795) and
10 an antimicrobial agent test strain at 50 μ l and 100
11 μ l.

12

13

14

15

Table 10: ODs at 16 hours of *E. coli* Cultures used as the Inoculum for the Multi-Strain Broth Test

<i>E.coli</i> test strain	Optical density at 590 nm (absorbance units)
25922	0.852
700728	0.423
12795	0.395

1

2

3

Table 11: Results of a multi-strain Trial of Test formulation

Time Post Inocul- ation (hours)	<i>E. coli</i> strain/Volume of Test formulation (μ l)									
	25922			700728			12795			
0	50	100	0	50	100	0	50	100	0	0
0	0.006	0.040	0.088	0.006	0.032	0.077	0.005	0.040	0.074	
1	0.034	0.021	0.031	0.020	0.030	0.028	0.017	0.024	0.035	
2	0.129	0.039	0.017	0.075	0.055	0.023	0.055	0.046	0.026	
3	0.263	0.074	0.018	0.197	0.106	0.013	0.133	0.101	0.024	
4	0.365	0.0138	0.011	0.335	0.188	0.019	0.311	0.198	0.044	
5	0.366	0.174	0.004	0.312	0.240	0.050	0.364	0.205	0.049	
6	0.395	0.234	0.009	0.371	0.264	0.044	0.399	0.264	0.038	
7	0.427	0.256	0.009	0.406	0.287	0.040	0.436	0.282	0.042	
23.5	0.688	0.351	0.049	0.545	0.323	0.096	0.564	0.296	0.079	
24.5	0.683	0.349	0.054	0.561	0.323	0.106	0.582	0.279	0.083	

4

1 Table 9 summarises the results of the 50 and 100
2 µl/100 ml broth test. These results indicated good
3 activity against *E. coli* 8739. Table 11 indicates
4 that the test formulation showed good activity when
5 challenged with other strains of *E. coli* including
6 two pathogenic strains 700728, 12795.

7

8 The 100 µl dose of the test formulation had the
9 lowest OD readings, therefore indicating greater
10 inhibition of cell proliferation. 50 µl/100 ml
11 broth of the test formulation appeared to have both
12 slowed cell proliferation and reduced the final
13 number of cells present in the broth. Where no
14 test formulation was present, growth was rapid for
15 all strains tested, especially in the first 4 hours
16 after inoculation.

17

18 The test formulation is only one of a range of
19 terpene formulations investigated so far and it is
20 clearly very active. Clear inhibition of *E. coli*
21 growth has been observed in broth tests conducted
22 at 50 µl and 100 µl/100 ml broth, both against anti
23 microbial assay strains and against pathogenic
24 strains.

25

26 Formulations have been developed now which show
27 very great activity against potentially lethal
28 strain 0157: H7 of *E. coli*, both at very high
29 innocula which are not sustainable in life and at
30 levels which, though likely to be fatal, are found.
31 Three 100 ml bottles were each filled with McConkey

1 broth to which was added one of either 20 µg/ml
2 oxacillin, or 10 µg/ml of amoxicillin, or 1 µg/ml
3 of the exemplary test terpene formulation. Each
4 bottle was then inoculated with 10^4 *E. coli*
5 0157:H:7 and incubated for 24 hours at 35°C.
6 Following incubation, the McConkey broth containing
7 the oxacillin had lost its magenta colour and
8 become yellowish and turbid, indicating that the
9 antibiotic had been overwhelmed by the *E. coli*. The
10 McConkey broth in the bottle containing the
11 amoxicillin had only slightly reduced magenta
12 colour, indicating that the antibiotic had
13 contained the *E. coli*, whereas the McConkey broth
14 in the bottle containing the terpene sample had an
15 undiminished magenta colour.

16

17 This experiment was then repeated under the same
18 conditions, except that the inoculum of *E. coli*
19 0157:H:7 was 10^8 . In this case, both the oxacillin
20 and amoxicillin samples were overwhelmed but the
21 McConkey broth in the bottle containing the terpene
22 sample had an undiminished magenta colour,
23 indicating that, even with this extremely high
24 inoculum, no growth had occurred.

25

26 Experiments have been carried out on xanthomonads
27 including assay strains such as *Xylella maltifolia*
28 and plant pathogens such as *X. fastidiosa*. The
29 latter causes Pierce's disease which has devastated
30 grape culture in Southern California and threatens
31 the wine growing areas of Napa Valley and Sonoma

1 Valley. The organisms are highly susceptible to
2 terpene formulations according to the present
3 invention.

4

5 It will be apparent for those skilled in the art
6 that a number of modifications and variations may
7 be made without departing from the scope of the
8 present invention as set forth in the appending
9 claims.

10

1 References

2

3 1. Bae EA, MJ Han, NJ Kim and DH Kim, 1998.

4 Anti-Helicobacter pylori activity of herbal
5 medicines. Biol, Pharm. Bull 21(9) 990-992.

6

7 2. Bard, M, MR Albert, N Gupta, CJ Guuynn and W
8 Stillwell, 1988. Geraniol interferes with membrane
9 functions in strains of Candida and Saccharomyces.
10 Lipids 23(6) : 534-538.

11

12 3. Barranx A, M Barsacq, G Dufau and JP Lauilhe,
13 1998. Disinfectant or antiseptic composition
14 comprising at least one terpene alcohol and at
15 least one bactericidal acidic surfactant, and use
16 of such a mixture. US patent# 5763468.

17

18 4. Boyanova L and G Neshev, 1999. Inhibitory effect
19 of rose oil products on Helicobacter pylori growth
20 in vivo: preliminary report. J. Med. Microbiol. 48:
21 705-706.

22

23 5. Chaumont JP and D Leger, 1992. Campaign against
24 allergic moulds in dwellings. Inhibitor properties
25 of essential oil geranium "Bourbon", citronellol,
26 geraniol and citral. Ann Pharm Fr 50(3): 156-166.

27

28 6. Crowell, PL and MN Gould, 1994. Chemoprevention
29 and therapy of cancer by d-limonene. Crit Rev Oncog
30 5(1): 1-22.

31

- 1 7. Crowell, PL, S Ayoubi and YD Burke, 1996.
- 2 Antitumorigenic effects of limonene and perillyl
- 3 alcohol against pancreatic and breast cancer. Adv
- 4 Exp Med Biol 401: 131-136.
- 5
- 6 8. Dupont H.L., C.D. Ericsson, J.J. Mathewson, M.W.
- 7 Dupont, Z.D. Jiang, A. Mosavi and F.J. de la
- 8 Cabana, 1998. Rifaximin: a nonabsorbed
- 9 antimicrobial in the therapy of traveler's
- 10 diarrhea. Digestion 59: 708-714.
- 11
- 12 9. Elegbede, JA, CE Elson, A Qureshi, MA Tanner and
- 13 MN Gould, 1984. Inhibition of DMBA-induced mammary
- 14 cancer by monoterpene d-limonene. Carcinogenesis
- 15 5(5): 661-664.
- 16
- 17 10. Elegbede, JA, CE Elson, A Qureshi, MA Tanner
- 18 and MN Gould, 1986. Regression of rat primary
- 19 mammary tumors following dietary d-limonene. J Natl
- 20 Cancer Inst. 76(2): 323-325.
- 21
- 22 11. Elson, CE and SG Yu, 1994. The chemoprevention
- 23 of cancer by mevalonate-derived constituents of
- 24 fruits and vegetables. J Nutr. 124: 607-614.
- 25
- 26 12. Ericsson, Charles, 1998. Traveler's diarrhea:
- 27 Epidemiology, prevention and self-treatment. Travel
- 28 Medicine 12 (2): 285-303.
- 29
- 30 13. Grubel P and DR Cave, 1998. Sanitation and
- 31 houseflies (*musca domestica*):factors for the

- 1 transmission of Helicobacter pylori. Bull. Inst.
- 2 Pasteur 96: 83-81.
- 3
- 4 14. Hooser, SB, VR Beasley and JJ Everitt, 1986.
- 5 Effects of an insecticidal dip containing dlimonene
- 6 in the cat. J Am Vet Med Assoc. 189(8): 905-908.
- 7
- 8 15. Ishii, E., 1993. Antibacterial activity of
- 9 teprenone, a non water-soluble antiulcer agent,
- 10 against Helicobacter pylori. Int. J Med Microbiol
- 11 Virol. Parasitol. Infect Dis. 280(12): 2391 243.
- 12
- 13 16. Iyer LM, JR Scott, and DF Whitfield, 1999.
- 14 Antimicrobial compositions. US patent 5,939,050.
- 15
- 16 17. Kadota S, P Basnet, E Ishii, T Tamura and T
- 17 Namba, 1997. Antibacterial activity of trichorabdal
- 18 A from Rabdosia trichocarpa against Helicobacter
- 19 pylori. Zentralbl. Bakteriol 287(1): 63-67.
- 20
- 21 18. Karlson, J, AK Borg, R Unelius, MC Shoshan, N
- 22 Wilking, U Ringborg and S Linda, 1996. Inhibition
- 23 of tumor cell growth by monoterpenes in vitro:
- 24 evidence of a Ras-independent mechanism of action.
- 25 Anticancer Drugs 7(4): 422-429.
- 26
- 27 19. Kim J, M Marshall and C Wei, 1995.
- 28 Antibacterial activity of some essential oil
- 29 components against five foodborne pathogens. J
- 30 Agric Food Chem. 43: 2839-2845.
- 31

- 1 20. Koga, J, T Yamauchi, M Shimura, Y Ogasawara, N
- 2 Ogasawara and J Suzuki, 1998. Antifungal terpene
- 3 compounds and process for producing the same. US
- 4 patent# 5,849,956.
- 5
- 6 21. Kubo J, JR Lee and I Kubo, 1999.
- 7 Anti-Helicobacter pylori agents from the cashew
- 8 apple. J Agric Food Chem. 47: 533-537.
- 9
- 10 22. Iyer LM, JR Scott, and DF Whitfield, 1999.
- 11 Antimicrobial compositions. US patent# 5,939,050.
- 12
- 13 23. Mikhlin ED, VP Radina, AA Dmitrossky. LP
- 14 Blinkova and LG Button, 1983. Antifungal and
- 15 antimicrobial activity of some derivatives of
- 16 beta-ionone and vitamin A. Prikl Biokhim Mikrobiol.
- 17 19: 795-803.
- 18
- 19 24. Moleyar V and P Narasimham, 1992. Antibacterial
- 20 activity of essential oil components. Int J Food
- 21 Microbiol 16(4): 337-342.
- 22
- 23 25. Onawunmi, GO, 1989. Evaluation of the
- 24 antimicrobial activity of citral. Letters in
- 25 Applied Microbiology 9(3): 105-108.
- 26
- 27 26. Pattnaik, S, VR Subramanyan, M Bapaji and CR
- 28 Kole, 1997. Antibacterial and antifungal activity
- 29 of aromatic constituents of essential oils.
- 30 Microbios 89(358): 39-46.
- 31

- 1 27. Petschow BW, RP Batema and LL Ford, 1996.
- 2 Susceptibility of Helicobacter pylori to
- 3 bactericidal properties of medium-chain
- 4 monoglycerides and free fatty acids. Antimicrobial
- 5 Agents and Chemotherapy 40 (2): 302-306.
- 6
- 7 28. Salt, SD, S Tuzun and J Kuc, 1986. Effects of
- 8 B-ionone and abscisic acid on the growth of tobacco
- 9 and resistance to blue mold. Mimicry the effects of
- 10 stem infection by Pero ospora tabacina. Adam
- 11 Physiol Molec Plant Path 28: 287-297.
- 12
- 13 29. Wright, DC, 1996. Antimicrobial oil-in-water
- 14 emulsions. US Patent #5,547,677.
- 15
- 16 30. Wright, DC, 1996. Antimicrobial oil-in-water
- 17 emulsions. US Patent #5,549,901.
- 18
- 19 31. Wright, DC, 1997. Antimicrobial oil-in-water
- 20 emulsions. US Patent #5,618,840.
- 21
- 22 32. Wright, DC, 1997. Micellar nanoparticles. US
- 23 Patent #5,629,021.
- 24
- 25 33. Wright, DC, 1997. Oil containing lipid vesicles
- 26 with marine applications. US Paten #5,662,957.
- 27
- 28 34. Wright, DC, 1997. Lipid vesicles having a
- 29 bilayer containing a surfactant with anti-viral and
- 30 spermicidal activity. US Patent #5,700,679.

1 35. Wright, DC, 1998. Oral vaccine against gram
2 negative bacterial infection. US Patent #5,730,989.

3

4 36. Yu, SG, PJ Anderson and CE Elson, 1995. The
5 efficacy of B-ionone in the chemoprevention of rat
6 mammary carcinogenesis. J Agri Food Chem 43:
7 2144-2147.

8

9

1 CLAIMS
2

3 1. An antimicrobial composition for preventing or
4 treating digestive tract infections, said
5 composition comprising a terpene or a mixture of
6 two or more terpenes.

7

8 2. The composition as claimed in Claim 1 which
9 comprises a mixture of the terpenes carvone and
10 geraniol.

11

12 3. The composition as claimed in either one of
13 Claims 1 and 2 which further comprises a
14 surfactant.

15

16 4. The composition of Claim 3 which consists of 1 to
17 99% terpenes and 1 to 99% surfactant.

18

19 5. The composition as claimed in either one of
20 Claims 3 and 4 wherein the terpene or terpene
21 mixture are natural or synthetic terpenes
22 selected from citral, b-ionone, geraniol,
23 carvacrol, eugenol, carvone, terpeniol, anethole
24 or other generally recognized as safe terpenes
25 with biocidal properties, and the surfactant is
26 selected from polysorbate-80, polysorbate-20,
27 polysorbate-40, polysorbate-60, polyglyceryl
28 esters, polyglyceryl monooleate, decaglyceryl
29 monocaprylate, propylene glycol dicaprilate,
30 triglycerol monostearate or their combination.

31

32

1 6. The composition as claimed in any one of Claims
2 1 to 5 wherein the terpene or terpene mixture is
3 at least partially encapsulated in a liposome to
4 form a liposome-terpene(s) combination.

5

6 7. The composition as claimed in Claims 1 to 6
7 wherein the terpene, terpene mixture or the
8 liposome-terpene(s) combination is part of the
9 inner core of a gelatin or cellulose capsule.

10

11 8. The composition as claimed in Claims 1 to 6
12 wherein the terpene, terpene mixture or the
13 liposome-terpene(s) combination is freeze dried,
14 spray dried or dried in order to form a powder
15 for encapsulation or solubilization.

16

17 9. The composition as claimed in Claims 1 to 6
18 wherein the terpene, terpene mixture or the
19 liposome-terpene(s) combination is freeze dried,
20 spray dried or dried in order to be compressed
21 in pill or tablet form.

22

23 10. The composition as claimed in Claims 1 to 6
24 wherein the terpene, terpene mixture or the
25 liposome-terpene(s) combination is freeze dried,
26 spray dried or dried in order to be compressed
27 in pill/tablet and coated for absorption in
28 different areas along the gastro-intestinal
29 tract.

30

31 11. A method to prevent or treat microbial
32 infections of the digestive tract, said method

- 1 comprising orally administering a composition as
2 claimed in any one of Claims 1 to 10 to patient.
- 3 12. The method as claimed in Claim 11 wherein the
4 digestive tract infections are produced by
5 normal, pathogenic or opportunistic
6 microorganisms or its toxins selected from
7 *Aerobacter sp.*, *Aspergillus sp.*, *Bacillus sp.*,
8 *Campylobacter sp.*, *Candida sp.*, *Clostridia sp.*,
9 *Enterobacteriaceae sp.*, *Enterococcus sp.*,
10 *Escherichia sp.*, *Haemophilus sp.*, *Helicobacter*
11 *sp Klebsiella sp.*, *Lactobacillus sp.*, *Listeria*
12 *sp.*, *Propionibacter sp.*, *Pasteurella sp.*,
13 *Proteus sp.*, *Pseudomonas sp.*, *Salmonella sp.*,
14 *Shigella sp.*, *Staphylococcus sp.*, *Streptococcus*
15 *sp.* and *Yersennia sp.*
- 16
- 17 13. The method as claimed in Claim 11 wherein the
18 terpene, terpene mixture or the
19 liposome-terpene(s) combination is effective
20 against pathogenic and normal microflora
21 comprising of *Aerobacter sp.*, *Aspergillus sp.*,
22 *Bacillus sp.*, *Campylobacter sp.*, *Candida sp.*,
23 *Clostridia sp.*, *Enterobacteriaceae sp.*,
24 *Enterococcus sp.*, *Escherichia sp.*, *Haemophilus*
25 *sp.*, *Helicobacter sp Klebsiella sp.*,
26 *Lactobacillus sp.*, *Listeria sp.*, *Propionibacter*
27 *sp.*, *Pasteurella sp.*, *Proteus sp.*, *Pseudomonas*
28 *sp.*, *Salmonella sp.*, *Shigella sp.*,
29 *Staphylococcus sp.*, *Streptococcus sp.* and
30 *Yersennia sp.*
- 31

1 14. The method as claimed in Claim 11 wherein the
2 terpene, terpene mixture or the
3 liposome-terpene(s) combination is effective
4 against pathogenic and opportunistic
5 microorganisms causing traveler's diarrhea.

6

7 15. The method as claimed in Claim 11 wherein the
8 terpene, terpene mixture or the
9 liposome-terpene(s) combination is effective
10 against pathogenic and opportunistic
11 microorganisms causing ulcers along the
12 digestive tract.

13

14 16. The method as claimed in Claim 11 wherein the
15 terpene, terpene mixture or the
16 liposome-terpene(s) combination is effective
17 against anthrax.

18

19 17. The method as claimed in Claim 11 wherein the
20 terpene, terpene mixture or the
21 liposome-terpene(s) combination is effective
22 against pathogenic and opportunistic
23 microorganisms causing scours in calves.

24

25 18. The method as claimed in Claim 11 wherein the
26 terpene, terpene mixture or the
27 liposome-terpene(s) combination is effective
28 against pathogenic and opportunistic
29 microorganisms causing scours in neonates and
30 weaned piglets.

31

1 19. The method as claimed in Claim 11 wherein the
2 terpene, terpene mixture or the
3 liposome-terpene(s) combination at lower
4 concentrations has a bacteriostatic effect
5 against pathogenic and normal gastro-intestinal
6 microflora.

7

8 20. The method as claimed in Claim 11 wherein the
9 terpene, terpene mixture or the
10 liposome-terpene(s) combination at higher
11 concentrations has a bactericidal effect against
12 pathogenic and normal gastro-intestinal
13 microflora.

14

15 21. The method as claimed in any one of Claims 11 to
16 20 wherein the effective dose of the terpene,
17 the mixture of terpenes or the
18 liposome-terpene(s) combination is between 20 mg
19 and 5000 mg.

20

21 22. The method as claimed in any one of Claims 11 to
22 20 wherein the effective dose of the terpene,
23 the mixture of terpenes or the
24 liposome-terpene(s) combination is between 20
25 ppm and 50000 ppm in water and/or food consumed
26 by the human or animal.

27

28 23. The method as claimed in any one of Claims 11 to
29 22 wherein the terpene, the mixture of terpenes
30 or the liposome-terpene(s) combination is
31 prepackaged in liquid form for oral consumption
32 by humans or animals.

1 24. The method as claimed in any one of Claims 11 to
2 23 wherein the terpene, a mixture of terpenes or
3 the liposome-terpene(s) combination is mixed
4 with milk replacer and fed to calves and
5 piglets.

6

7 25. The method as claimed in any one of Claims 11 to
8 24 wherein the terpene, the mixture of terpenes
9 or the liposome-terpene(s) combination is
10 intubated directly into the stomach of an
11 animal.

12

13

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 02/00015

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61K31/11 A61K31/045 // (A61K31/11, 31:045)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, CHEM ABS Data, EPO-Internal, PAJ, MEDLINE, WPI Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOYANOVA LUDMILA ET AL: "Inhibitory effect of rose oil products on Helicobacter pylori growth in vitro: Preliminary report." JOURNAL OF MEDICAL MICROBIOLOGY, vol. 48, no. 7, July 1999 (1999-07), pages 705-706, XP002196283 ISSN: 0022-2615 the whole document	1-5
Y		6-10
X	WO 97 02040 A (BEVILACQUA MARIA ;MICHELIN LAUSAROT ELISA (IT); BEVILACQUA MATTEO) 23 January 1997 (1997-01-23) claims 1-4 page 8, line 9-12 page 11, line 25-30	1,3-5, 7-10
		-/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 April 2002	03/05/2002
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Herrera, S

INTERNATIONAL SEARCH REPORT

Int'l Application No
PCT/GB 02/00015

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 763 468 A (LAUILHE JEAN-PAUL ET AL) 9 June 1998 (1998-06-09) abstract example 3 ---	1,3-5
Y	DE 35 11 862 A (KLINGE CO CHEM PHARM FAB) 9 October 1986 (1986-10-09) page 6, line 9 -page 7, line 1; claims 22,23 ---	1-10
X	DE 35 11 862 A (KLINGE CO CHEM PHARM FAB) 9 October 1986 (1986-10-09) page 6, line 9 -page 7, line 1; claims 22,23 ---	1,3-5
Y	US 5 939 050 A (IYER LOKANATHAN M ET AL) 17 August 1999 (1999-08-17) claims ---	1-10
Y	PATENT ABSTRACTS OF JAPAN vol. 1997, no. 08, 29 August 1997 (1997-08-29) & JP 09 110683 A (LION CORP), 28 April 1997 (1997-04-28) abstract ---	1-10
X	PATENT ABSTRACTS OF JAPAN vol. 1999, no. 06, 31 March 1999 (1999-03-31) & JP 08 027017 A (DIMOTECH LTD), 30 January 1996 (1996-01-30) abstract ---	1,3-5

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l. Application No

PCT/GB 02/00015

Patent document cited in search report		Publication date		Patent family member(s)	Publication date
WO 9702040	A	23-01-1997		IT PD950133 A1 IT PD950134 A1 IT PD960038 A1 AU 6305896 A WO 9702040 A1 EP 0836478 A1	03-01-1997 03-01-1997 20-08-1997 05-02-1997 23-01-1997 22-04-1998
US 5763468	A	09-06-1998		FR 2727289 A1 AT 190197 T AU 4265196 A CA 2181940 A1 DE 69515468 D1 DE 69515468 T2 EP 0748162 A1 ES 2145313 T3 WO 9616548 A1	31-05-1996 15-03-2000 19-06-1996 06-06-1996 13-04-2000 26-10-2000 18-12-1996 01-07-2000 06-06-1996
DE 3511862	A	09-10-1986		DE 3511862 A1	09-10-1986
US 5939050	A	17-08-1999		AU 727242 B2 AU 6877198 A BR 9804815 A CA 2257500 A1 CN 1225585 T CZ 9803847 A3 EP 0934067 A1 HU 9903759 A2 JP 2000514834 T NO 985643 A NZ 333145 A SK 162998 A3 WO 9844926 A1 US 6248309 B1	07-12-2000 30-10-1998 25-01-2000 15-10-1998 11-08-1999 14-04-1999 11-08-1999 28-04-2000 07-11-2000 03-02-1999 28-10-1999 12-07-1999 15-10-1998 19-06-2001
JP 09110683	A	28-04-1997		NONE	
JP 08027017	A	30-01-1996		NONE	